Long-Term Inactivation Study of Three Enteroviruses in Artificial Surface and Groundwaters, Using PCR and Cell Culture[▽]

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Since the transmission of pathogenic viruses via water is indistinguishable from the transmission via other routes and since the levels in drinking water, although significant for health, may be too low for detection, quantitative viral risk assessment is a useful tool for assessing disease risk due to consumption of drinking water. Quantitative viral risk assessment requires information concerning the ability of viruses detected in drinking water to infect their host. To obtain insight into the infectivity of viruses in relation to the presence of virus genomes, inactivation of three different enteroviruses in artificial ground and surface waters under different controlled pH, temperature, and salt conditions was studied by using both PCR and cell culture over time. In salt-peptone medium, the estimated ratio of RNA genomes to infectious poliovirus 1 in freshly prepared suspensions was about 10^0. At 4°C this ratio was 10^3 after 600 days, and at 22°C it was 10^4 after 200 days. For poliovirus 1 and 2 the RNA/infectious virus ratio was higher in artificial groundwater than in artificial surface water, but this was not the case for coxsackievirus B4. When molecular detection is used for virus enumeration, it is important that the fraction of infectious virus (based on all virus genomes detected) decays with time, especially at temperatures near 22°C.

Worldwide, infectious diseases have been linked to consumption of drinking water (for reviews, see references 2 and 15). Like other infectious microorganisms, viruses may cause waterborne diseases that are more serious than self-limiting gastroenteritis, such as meningitis and poliomyelitis, and eventually in some cases may cause death. Viruses may be of concern since they may be more resistant than bacteria to inactivation by both natural processes, such as sunlight (25), and drinking water treatment (e.g., UV treatment) (11). Moreover, viruses may form recombinant species as a result of exposure of their hosts to environmentally contaminated water or food-stuffs containing multiple strains of the same virus type (21). In addition, viral pathogens have a low dose-response relationship for infection and infectious disease in humans. Even at low levels that cannot be directly detected in drinking water, the infectious agents can cause waterborne disease. However, pathogens may be detected in source waters used for production of drinking water as required by Dutch law (http://www.vrom.nl). Taking into account reductions in the numbers of pathogens in these source waters due to treatment of drinking water, pathogen counts in drinking water may be estimated. Several characteristics of the pathogens must be known to assess the health risk when humans are exposed to viruses in drinking water, such as the type, count, and infectivity. In addition to early studies of degradation of the coat protein that provided evidence concerning nucleic acid infectivity (23), the infectivity of viruses may be determined by using cell cultures,

such as the well-known Buffalo green monkey kidney (BGM) cell line for enteroviruses (5) and Caco-2 cells for rotaviruses (19). However, some of the most important waterborne viruses, such as hepatitis E viruses, are very difficult to culture with cell lines (27), and for other viruses, such as noroviruses, no sensitive cell line has been identified (6). Both hepatitis E viruses and noroviruses may be detected by molecular methods, such as PCR (13, 14). Although detection by PCR reveals the presence of viral RNA or DNA, it does not indicate the infectivity of the virus. Because of this the infection or disease risk due to consumption of drinking water may be overestimated. The detected genomic material may be present in otherwise defective virus particles that are not able to bind to or replicate in the host cells. In order to assess the infection or disease risk for these noncultivable pathogenic viruses, assumptions need to be made based on so-called PCR-detectable units. Here, we studied inactivation of three different viruses in artificial ground and surface waters under different controlled pH, temperature, and salt conditions as detected by using both PCR and cell cultures to determine whether the ratio of infectious particles to defective particles changed over time. Our data should help with quantitative assessment of the microbial risk for consumption of drinking water.

MATERIALS AND METHODS

Viruses. Inactivation experiments were conducted using three virus isolates, coxsackievirus type B4, which was originally obtained from the "Voorns kanaal" by using BGM cell culture, and poliovirus 1 and poliovirus 2 (Sabin). The virus stock suspensions consisted of supernatants of infected cell cultures. The infected cells were freeze-thawed once, and the cell debris was pelleted using low-speed centrifugation (1,000 × g). Virus titers were determined by the plaque assay method, as described below. Viruses were seeded using different concentrations in different storage media, depending on the experiment.

Storage medium. HBSP contained Hanks' balanced salt solution (Invitrogen, Leek, The Netherlands) and 50 g/liter peptone (Oxoid, Basingstoke, United

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TABLE 1. Storage conditions for the three experiments[a]

Expt	Storage medium(a)	Virus(es)	Virus concn (particles/ml)	No. of virus dilutions	Storage temp (°C)	Storage time (days)	No. of time points
1	HBSP	PV1	0–2,000	13 (twofold serial)	4 and 22	0–606	9
2	AGW	PV1 and CxB4	1–12	6	4, 10, and 22	7	1
3	AGW and ASW	PV1, PV2, and CxB4	0–240	6 (threefold serial)	4 and 22	0–342	8

[a] Abbreviations: PV1, poliovirus 1; CxB4, coxsackievirus B4; PV2, poliovirus 2; AGW, artificial groundwater; ASW, artificial surface water.

Kingdom). The properties of artificial groundwater and artificial surface water were adjusted so that they were similar to the properties of natural waters in The Netherlands. Artificial groundwater contained 35 mg $MgSO_4 \cdot 7H_2O$, 12 mg $CaSO_4 \cdot 2H_2O$, 12 mg $NaHCO_3$, 6 mg NaCl, and 6 mg KNO_3 per liter of ultrapure water with a pH of 7.0. Artificial surface water contained 101.4 mg $MgSO_4 \cdot 7H_2O$, 300.7 mg $CaSO_4 \cdot 2H_2O$, and 234 mg $NaHCO_3$ per liter of ultrapure water with a pH of 8.1.

Experiments. Three separate experiments were performed with different viruses and under different conditions (Table 1).

(i) Experiment 1. Seven series of flasks were filled with virus dilutions (sixfold) and were stored in the dark at 4 or 22°C. At each analysis time six flasks at each temperature were examined by using cell culture, and 100 μl from one flask was used for RNA extraction. During the experiment little inactivation was observed; therefore, selected dilutions were prepared and were tested either three times or once. During the whole experiment, the extracted RNA was tested six times.

(ii) Experiment 2. Three series of flasks were filled with virus dilutions and were stored in the dark at 4, 10, or 22°C. After 7 days of storage the dilutions were tested by using a plaque assay.

(iii) Experiment 3. Fifteen series of flasks were filled with virus dilutions (sixfold) and were stored in the dark at 4 or 22°C. At each analysis time two flasks were tested by using cell culture, and 100 μl from one flask was used for RNA extraction. The extracted RNA was tested four times.

RNA extraction. RNA was extracted from 100 μl of each virus dilution by binding to silica beads in the presence of a high-molarity solution (3). Briefly, 500 μl of lysis buffer L6 and 10 μl of a silica suspension were added to a virus dilution. The sample was mixed on a rotary shaker and then centrifuged briefly to pellet the silica particles. The pellet was washed twice with guanidinium thiocyanate-containing wash buffer L2 (3), twice with 70% (vol/vol) ethanol, and once with acetone. After the acetone was removed by evaporation, the RNA was eluted in 30 or 40 μl distilled water with RNAguard (200 U/ml; Pharmacia) and dithiothreitol (3 mM; Sigma, Zwijndrecht, The Netherlands) and was used in a reverse transcriptase PCR (RT-PCR).

RT-PCR. For enterovirus detection by RT-PCR, the highly conserved 5′ untranslated region was used as the target area. The RT-PCR was performed as described previously (24), with slight modifications. Briefly, 5 μl RNA was added to a mixture (final volume, 15 μl) containing 50 pmol 3′ primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, and 5 U of avian myeloblastoma virus RT (Boehringer Mannheim, Almere, The Netherlands). The mixture was incubated at 42°C for 1 h, heated for 5 min at 94°C to denature the enzyme, and then placed on ice. Five microliters of the RT-PCR mixture was added to a PCR mixture containing 10 mM Tris-HCl (pH 9.2), 75 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 2.5 U AmpliTaq (Perkin Elmer, Nieuwerkerk aan den IJssel, The Netherlands), and 20 pmol 5′ primer. Mineral oil was added, and 40 amplification cycles, each consisting of 1.5 min at 95°C, 1.5 min at 55°C, and 1.5 min at 72°C, were performed.

Gel electrophoresis, Southern blotting, and hybridization. The amplification products were analyzed by electrophoresis in 2% agarose gels and were visualized with UV illumination after staining with SYBR gold nucleic acid gel stain (Molecular Probes, Leiden, The Netherlands). The PCR products in the agarose gel were transferred to a positively charged nylon membrane (Amersham, 's-Hertogenbosch, The Netherlands) by using a vacuum blotting system (Millipore, Etten-Leur, The Netherlands) with 0.5 M NaOH (Merck, Amsterdam, The Netherlands) and 0.6 M NaCl (Merck, Amsterdam, The Netherlands) for 30 min. The specificity of the RT-PCR products of both viruses detected was confirmed as described previously (24).

Cell culture. Virus infectivity was determined by a monolayer plaque assay (22). Briefly, BGM cells were grown in confluent monolayers in 75-cm² plastic flasks. The culture medium was removed, and then an eluate and antibiotic mixture were added to the flasks. The cultures were incubated at room temperature for 120 min to allow virus adsorption to the cells. The cells were overlaid with medium 199 with Earle's salts (Life Technologies, Breda, The Netherlands) supplemented with 10% fetal bovine serum (Life Technologies), 0.9% Bacto agar (Difco, Amsterdam, The Netherlands), 0.2% bicarbonate, 100 IU penicillin, and 100 μg/ml streptomycin (Life Technologies). After 5 or 6 days of incubation at 37°C, the cells were stained with 0.03% neutral red in 0.9% agar. After 24 h the plaques were enumerated.

Statistical analysis. Virus concentrations were estimated based on the presence or absence of RT-PCR signals in serial dilutions of virus suspensions or based on plaque counts, assuming that the numbers of virus particles were Poisson distributed. Maximum likelihood concentrations and 95% confidence intervals were estimated from the Poisson likelihood for either counted plaques or presence-absence data, accounting for equivalent sample volumes (28).

Decay of either infectious virus or the RT-PCR-detected virus concentration $c(t)$ was modeled as biphasic:

$$c(t) = c_0[we^{-\lambda_1 t} + (1 - w)e^{-\lambda_2 t}]$$

where c_0 is the concentration at time zero, λ_1 and λ_2 are decay rates, and w is a mixing parameter. For an w value of 1 this function represents monophasic (log-linear) decay.

For an observed count (n) in an equivalent volume (V_{eq}) at time T, the contribution to the likelihood is

$$l(c(T)|n, V_{eq}) = \frac{e^{-c(T)V_{eq}}(c(T)V_{eq})^n}{n!}$$

where $c(T)$ is the biphasic regression function. The absence of virus (RT-PCR signal) in k replicate samples at a dilution corresponding to V_{eq} and the presence in m replicates at time T contributes the following term to the likelihood function

$$l(c(T)|k, m, V_{eq}) = (e^{-c(T)V_{eq}})^k(1 - e^{-c(T)V_{eq}})^m$$

The goodness of fit of biphasic decay functions versus monophasic decay functions ($w = 1$) was evaluated using a likelihood ratio test comparing the difference in -2 times the maximum log likelihood values to a chi-square statistic with 2 degrees of freedom ($4 - 2 = 2$). If the decrease in deviance of the biphasic decay model compared to the monophasic model was not significant, the latter model was accepted as the more parsimonious model. A similar likelihood ratio test was used to test for absence of virus decay ($\lambda_1 = 0$ in a monophasic model). Confidence intervals for predicted virus concentrations were obtained by Markov chain Monte Carlo sampling from the likelihood functions (8, 20). Ratios of RT-PCR-based virus to infectious virus (plaque count based) were calculated using the ratios of the estimated concentrations.

RESULTS

In this study the ratio of infectious particles to defective particles was studied under controlled conditions that varied with respect to salt concentration, pH, temperature, and virus type by using regression modeling PCR and cell culture data.

Poliovirus 1 inactivation in salt-peptone medium. Preservative medium was inoculated with poliovirus 1 that was serially twofold diluted to obtain 12 dilutions. Separate vials stored at either 4 or 22°C were examined by using PCR and cell culturing on days 0, 7, 14, 21, 53, 74, 88, 116, 179, 606, and 1022. For

TABLE 2. Results of cell culture and RT-PCR assays for day 0 and for day 7 at 4 and 22°C

Sample	Day 0							Day 7 at 4°C							Day 7 at 22°C						
	No. of PFU/1.1 ml						No. of RT-PCR-positive reactions	No. of PFU/1.1 ml						No. of RT-PCR-positive reactions	No. of PFU/1.1 ml						No. of RT-PCR-positive reactions
	Vial 1	Vial 2	Vial 3	Vial 4	Vial 5	Vial 6		Vial 1	Vial 2	Vial 3	Vial 4	Vial 5	Vial 6		Vial 1	Vial 2	Vial 3	Vial 4	Vial 5	Vial 6	
None	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	1	1	1	2	0	4	1	2	1	3	2	1	2	2	2	3	2	2	1	1
2	3	3	4	4	4	3	2	2	4	3	4	3	4	2	1	1	2	2	2	2	0
3	4	9	2	3	7	3	3	2	5	7	6	9	2	2	4	4	5	2	4	4	3
4	12	10	11	10	11	12	5	13	10	9	11	8	8	6	10	8	4	9	11	10	6
5	NCa	NC	NC	NC	NC	NC	6	NC	NC	NC	NC	NC	NC	6	12	NC	NC	NC	NC	11	6
6	NC	NC	NC	NC	NC	NC	6	NC	NC	NC	NC	NC	NC	6	NC	NC	NC	NC	NC	NC	6
7	NC	NC	NC	NC	NC	NC	6	NC	NC	NC	NC	NC	NC	6	NC	NC	NC	NC	NC	NC	6
8	NC	NC	NC	NC	NC	NC	6	NC	NC	NC	NC	NC	NC	6	NC	NC	NC	NC	NC	NC	6
9	NC	NC	NC	NC	NC	NC	6	NC	NC	NC	NC	NC	NC	6	NC	NC	NC	NC	NC	NC	6
10	NC	NC	NC	NC	NC	NC	6	NC	NC	NC	NC	NC	NC	6	NC	NC	NC	NC	NC	NC	6
11	NC	NC	NC	NDb	ND	ND	6	NC	NC	NC	ND	ND	ND	6	NC	NC	NC	ND	ND	ND	6
12	NC	NC	NC	ND	ND	ND	6	NC	NC	NC	ND	ND	ND	6	NC	NC	NC	ND	ND	ND	6

a NC, not countable.
b ND, not done.

example, six vials containing the lowest virus concentration were removed on day 0 for testing. For each vial 1.1 ml was directly cultured on BGM cells, which resulted in 0, 1, 1, 1, 2, and 0 PFU. Also, 100 μl from one of these six vials containing 1.2 ml was subjected to RNA extraction and subsequent RT-PCR, which resulted in four of six positive reactions after hybridization of the PCR products. This was done for every virus dilution. The control sample without added virus was negative as determined by cell culturing and PCR, as observed in each of the tests during the experiment. Similarly, the vials containing less diluted virus stock were each tested by using cell culturing and PCR; all data are shown for day 0 and for day 7 and 4 and 22°C are shown in Table 2.

As shown in Fig. 1, data obtained for 4 and 22°C were analyzed by regression modeling. On day 0 the number of infectious polioviruses determined by using cell culture was estimated to be 2,000 PFU/ml, compared with 20,000 PCR-detectable units per ml. Over time, little inactivation of poliovirus RNA was observed in the salt-peptone medium at 4 and 22°C, as determined by PCR (Fig. 1a and b, respectively). A biphasic trend was observed for poliovirus RNA inactivation at 22°C (Fig. 1b), as well as for infectious poliovirus at 4°C (Fig. 1a). The numbers of infectious polioviruses declined more rapidly at 22°C than at 4°C (Fig. 1b and a, respectively). The resulting ratio of poliovirus RNA genomes to infectious polioviruses on day 0 was approximately 10 (Fig. 1c and d).
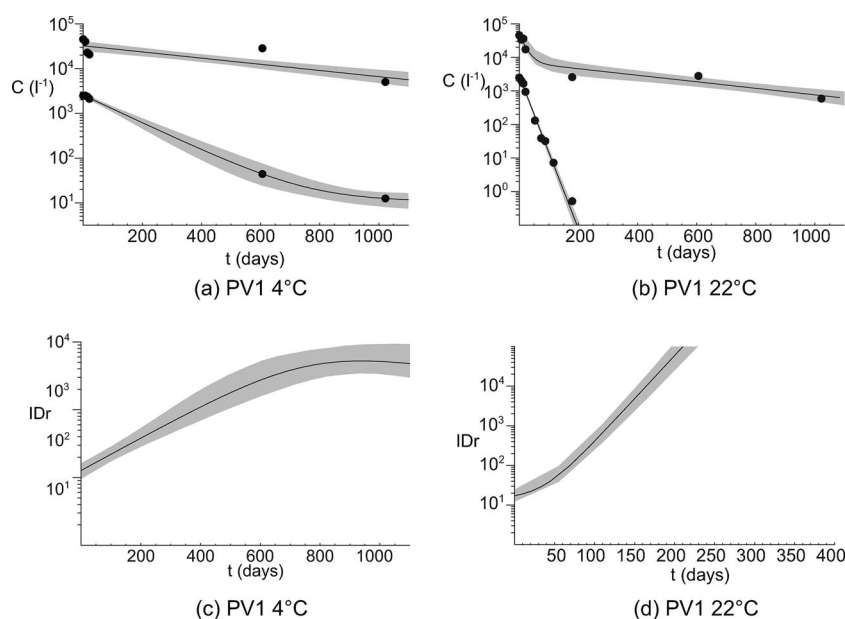

FIG. 1. Decay of virus concentrations (C) as determined by plaque assays and RT-PCR over a prolonged time period (t) at 4°C (a) and 22°C (b). (c and d) Ratios of PCR- and cell culture-based virus concentration estimates (IDr) at 4°C (c) and 22°C (d).
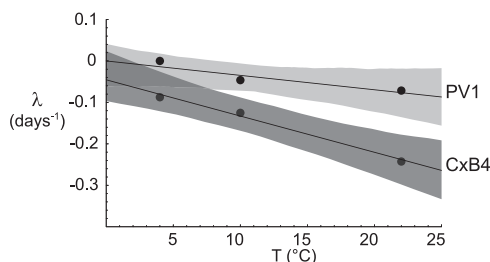
FIG. 2. Decay of virus concentration as determined by plaque assays as a function of storage temperature (T) for poliovirus 1 (PV) and coxsackievirus B4 (CxB4).

Poliovirus 1 and coxsackievirus B4 inactivation in artificial groundwater. Since little inactivation was observed in the preservative salt-peptone medium, a pilot cell culture experiment was conducted with artificial groundwater at neutral pH (pH 7.0) with average salinity; 10°C was added to the temperature range to mimic groundwater temperatures in The Netherlands. Moreover, in addition to poliovirus 1, another enterovirus, a coxsackievirus B4 strain originally isolated from a Dutch canal, was included in the experiment. Indeed, a much higher rate of decay of infectious poliovirus was observed in artificial groundwater than in the preservative medium. For coxsackievirus B4

the decay rate was slightly lower and was not significantly different from that of poliovirus 1 over a limited time period (6 days) (Fig. 2). A linear decrease (a single decay rate) in the virus concentrations was observed for both enteroviruses at temperatures from 4°C to 10°C to 22°C.

Comparison of virus inactivation under different pH, temperature, and salt conditions. Human volunteer studies have revealed differences between dose-response relationships for different enteroviruses, not only in comparisons of enterovirus types but also in comparisons of strains and variants (29). In the experiment described above there was less inactivation of coxsackievirus type B4 than of poliovirus 1 in a short time period. In this experiment not only were poliovirus 1 and coxsackievirus B4 tested, but also poliovirus 2 was included, and the study period was extended from 6 to 342 days.

At 4°C, the decay rate was similar, although somewhat higher, for infectious enteroviruses compared with enterovirus genomes in artificial groundwater (Fig. 3a, c, and e). However, infectious enteroviruses were inactivated at a much higher rate than enterovirus genomes at 22°C (Fig. 3b, d, and f). At this temperature, coxsackievirus B4 was no longer detected in artificial groundwater by using cell cultures after 110 days, whereas polioviruses 1 and 2 were not detectable after 160 and 150 days, respectively (Fig. 3b, d, and f). In artificial surface water at 4°C, the decay rate was somewhat higher for infectious

(a) PV1 AGW 4°C

(b) PV1 AGW 22°C

(c) PV2 AGW 4°C

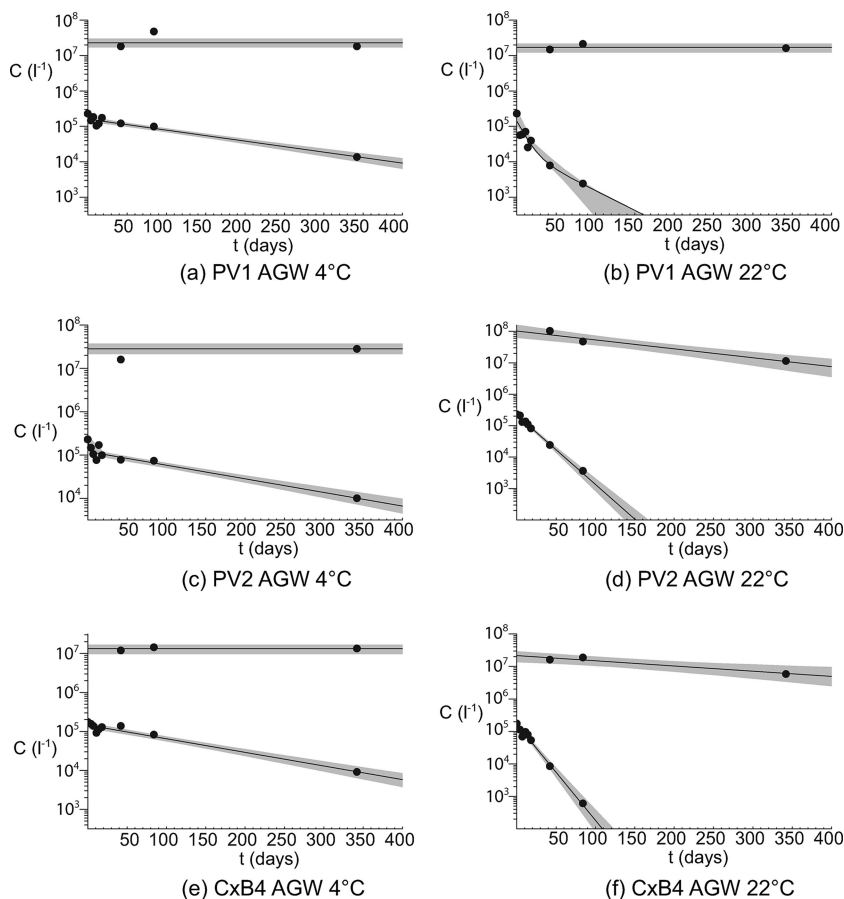(d) PV2 AGW 22°C

(e) CxB4 AGW 4°C

(f) CxB4 AGW 22°C

FIG. 3. Decay of virus concentrations (C) as determined by plaque assays and RT-PCR at 4°C (a, c, and e) and 22°C (b, d, and f) in artificial groundwater for poliovirus 1 (a and b), poliovirus 2 (c and d), and coxsackievirus B4 (e and f). t, time.
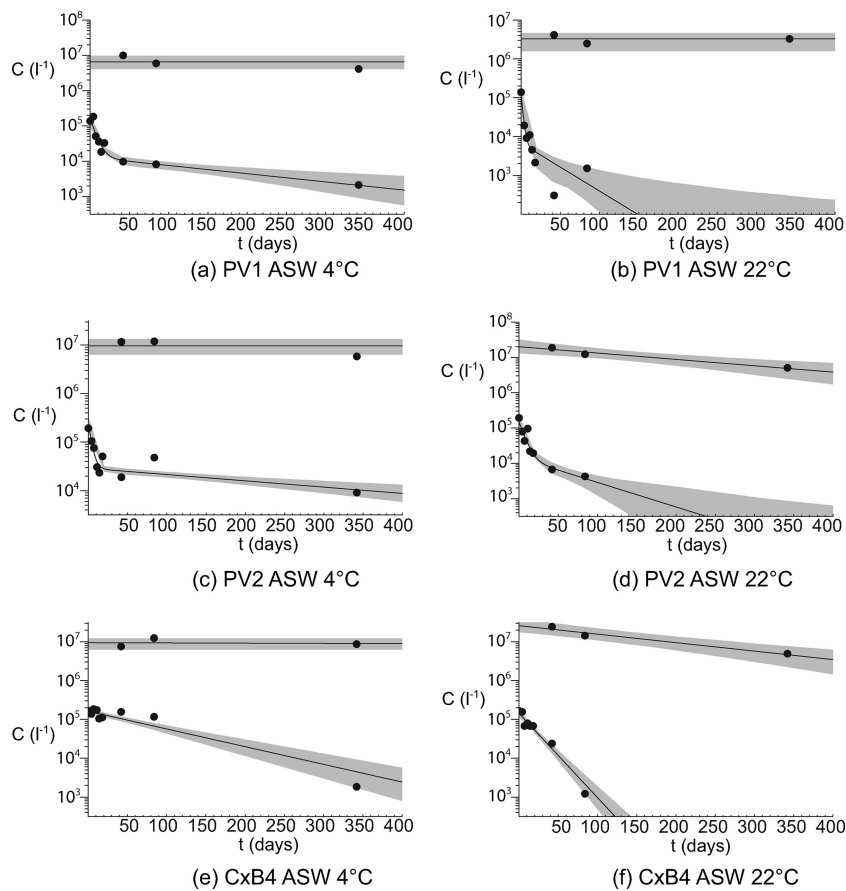
FIG. 4. Decay of virus concentrations (C) as determined by plaque assays and RT-PCR at 4°C (a, c, and e) and 22°C (b, d, and f) in artificial surface water for poliovirus 1 (a and b), poliovirus 2 (c and d), and coxsackievirus B4 (e and f). t, time.

enteroviruses than for enterovirus genomes (Fig. 4a, c, and e). A biphasic trend was observed for both polioviruses but not for coxsackievirus B4 (Table 3). At 22°C, infectious poliovirus 2 was not detected in artificial surface water after 240 days, compared with 150 and 120 days for poliovirus 1 and coxsack-

ievirus B4, respectively (Fig. 4b, d, and f). In contrast, entero-virus RNA genomes could be detected by PCR for up to 342 days whether they were in artificial surface water or ground-water or at 4 or 22°C (Fig. 3 and 4). As shown in the first experiment poliovirus 1 RNA genomes could be detected at

TABLE 3. Decay rates of poliovirus 1, poliovirus 2, and coxsackievirus B4 in artificial groundwater and artificial surface water at 4 and 22°C

Virus	Water[a]	Temp (°C)	Cell culture		RT-PCR	
			Decay rate (day^{-1})	Half time (days)	Decay rate (day^{-1})	Half time (days)
Poliovirus 1	AGW	4	0.0072	96.9	0	∞
		22	0.026[b]	26.9[b]	0	∞
Poliovirus 1	ASW	4	0.0054[b]	129.5[b]	0	∞
		22	0.028[b]	24.4[b]	0	∞
Poliovirus 2	AGW	4	0.0072[b]	95.9[b]	0	∞
		22	0.051	13.6	0.0065	106.0
Poliovirus 2	ASW	4	0.003[b]	231.2[b]	0	∞
		22	0.016[b]	43.5[b]	0.0041	167.9
Coxsackievirus B4	AGW	4	0.0081	85.9	0	∞
		22	0.068	10.2	0.0037	187.4
Coxsackievirus B4	ASW	4	0.010	66.3	0	∞
		22	0.050	13.9	0.0050	137.5

[a] ASW, artificial surface water; AGW, artificial groundwater.
[b] Biphasic decay. Only the long-term rate is shown.

TABLE 4. Ratio of RT-PCR-based virus concentration estimates to cell culture-based estimates based on decay curves at 4 and 22°C, extrapolated to time zero

Virus	Water[a]	Ratio
Poliovirus 1	AGW	117.9
	ASW	39.7
Poliovirus 2	AGW	164.0
	ASW	62.8
Coxsackievirus B4	AGW	99.5
	ASW	80.6

[a] ASW, artificial surface water; AGW, artificial groundwater.

both temperatures even after 1,022 days in medium (Fig. 1a and b).

In general, on day 0 the number of infectious enteroviruses determined by using cell cultures was estimated to be 10^5 PFU/ml, compared with 10^7 PCR detectable units per ml, resulting in a ratio of approximately 100 (Fig. 3 and 4). When water type was considered, coxsackievirus type B4 appeared to be the most stable, with a ratio of 99.5 in artificial groundwater and a ratio of 80.6 in artificial surface water (Table 4). The ratios for polioviruses 1 and 2 were higher for groundwater (117.9 and 164.0, respectively) than for surface water (39.7 and 62.8, respectively).

In artificial groundwater the changes in the ratio of virus RNA genomes to infectious virus due to decay occurred slowly at 4°C (about 10-fold per year) (Fig. 5a, e, and i) compared to the changes at 22°C, because of the rapid decay of infectious virus at the higher temperature.

In artificial surface water the same pattern was observed (Fig. 5), but at 4°C the ratios for polioviruses 1 and 2 appeared to show biphasic changes (Fig. 5c and g) due to biphasic decay of infectious virus (Fig. 4 a and c). After rapid initial decay the long-term changes in the ratio at 22°C were slower than those in groundwater for polioviruses (compare Fig. 5b and f and Fig. 5d and h). The changes in the ratio for coxsackievirus B4 in artificial groundwater and artificial surface water were similar (Fig. 5i, j, k, and l).

DISCUSSION

The extent to which defective virus is produced is affected by viral and host factors. Molecular methods used with water samples detect the viral genome, which is not indicative of the infectivity of the virus in the environment (7, 26). Public health risks are overestimated if molecular methods are used for virus enumeration since both infectious particles and defective particles are included, but for some viruses there are not alternative methods. In this study the ratio of defective particles to infectious particles was assessed under controlled conditions which were varied with respect to salt concentration, pH, temperature, and virus type by using regression modeling of PCR and cell culture data as input for quantitative viral risk assessment.
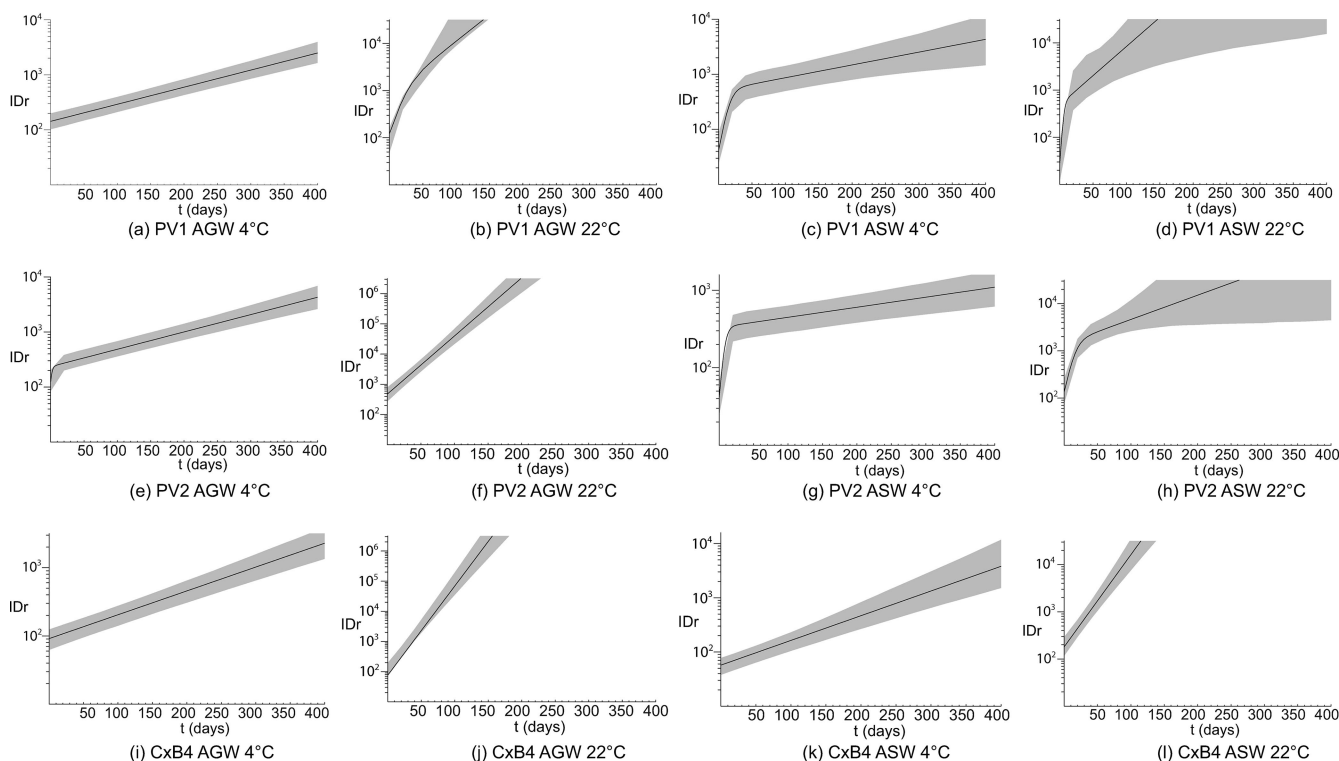

FIG. 5. (a, b, e, f, i, j) Ratios of RT-PCR- and cell culture-based virus concentration estimates (IDr) for 4°C (a, e, and i) and 22°C (b, f, and j) in artificial groundwater for poliovirus 1 (a and b), poliovirus 2 (e and f), and coxsackievirus B4 (i and j). (c, d, g, h, k, and l) Ratios of RT-PCR- and cell culture-based virus concentration estimates for 4°C (c, g, and k) and 22°C (d, h, and l) in artificial surface water for poliovirus 1 (c and d), poliovirus 2 (g and h), and coxsackievirus B4 (k and l). t, time.

Inactivation of poliovirus 1 RNA was much slower than inactivation of infectious poliovirus 1, especially at higher temperatures in preservative medium. The decay rate was higher for infectious enteroviruses than for enterovirus genomes in artificial ground and surface waters at 4°C, and this was even more true at 22°C. Combined RNA transfection and cell culture experiments also showed that compared with inactivation by UV or hypochlorite, thermal inactivation significantly changed virion and RNA infectivity (18). The rate of decay of infectious poliovirus 1 observed in artificial groundwater was much higher than the rate of decay in the preservative medium observed for coxsackievirus B4, which had a similar linear decay rate at a higher temperature. Yates et al. (31) showed that temperature was the only variable that was significantly correlated with decay of poliovirus 1, echovirus type 1, and MS2 in groundwater irrespective of the virus type (31).

In preservative medium the initial ratio (day 0) of the number of poliovirus 1 PCR units and the number of cell culture units was approximately 10, and this ratio increased to 1,000 at 4°C over more than 2 years and increased to infinity at 22°C because the concentration of infectious virus tends to zero. On day 0 the enterovirus ratio in artificial waters was approximately 100, which may have been explained by relatively rapid initial inactivation in artificial waters compared with preservative medium. In artificial waters temperature was not critical for the poliovirus 1 ratio, but the ratio was threefold higher for groundwater than for surface water. For coxsackievirus B4 there was no difference in the ratios between artificial groundwater and surface water. The greatest differences were observed for poliovirus 2, and the ratios varied from 50 to 500 depending on the temperature, as well as the pH and salt concentration. We established that the ratio of defective virus particles to infectious virus particles under controlled conditions depends on different factors related to virus type.

In natural circumstances additional factors determine the ratio of defective virus to infectious virus, such as the time after replication or age. Viruses enter source waters at different ages that are not known but influence the infectious state, as shown here. The important factors for virus reduction in water include the presence of indigenous microflora. It is known that compared with poliovirus 1 (used in our study), some enteroviruses, especially coxsackievirus A-9, are susceptible to proteolytic enzymes. These enzymes originate from proteolytic bacteria, such as *Pseudomonas aeruginosa* (4, 10), that are known to be present in surface waters in The Netherlands (30). The finding that virus inactivation was more rapid in a lake than in sterile lake water (10) was also significant. Studies of the role of the indigenous microflora in decay of virus infectivity need to be carried out in order to collect data for risk assessment. The artificial source waters used in our experiments did not include the indigenous microflora because the presence of the natural microflora would have caused additional heterogeneity that would have been difficult to control. Additional inactivation caused even more-rapid decay of the ratio of RT-PCR-determined virus to infectious virus. Therefore, for risk assessment our estimates of the ratio infectious virus to total virus can be treated as "best-case" estimates. A PCR negative signal does indicate that in a reliable test the specific virus type is not present in the volume of source water tested whether it is infectious or not. This may be useful for

assessing the risk of producing drinking water from this source water. In estimating the efficiency of treatment processes PCR may also be useful, but it may be more useful for physical processes, such as filtration, than for disinfection processes, such as UV inactivation. Other comparisons, such as the use of RT and PCR to discriminate between infectious and noninfectious hepatitis A virus, may also be useful for assessing treatment efficiency but are not useful for natural source waters (1).

Another important determinant in the detection of infectious virus particles is the fact that each of the enteroviruses has a different affinity for the cell line receptors, influencing the counts. For instance, some enteroviruses, such as coxsackievirus A1, do not grow at all on BGM cells (5), and for some viruses, such as the prevalent human caliciviviruses and noroviruses, no cell line that can sustain replication of the virus has been established so far (6). For viruses that cannot be cultured, PCR is one of the only detection methods available. PCR-detectable unit data can be extrapolated to infectious viruses that cannot be cultured in vitro based on the experiments with specific enteroviruses described here since the ratios observed in our experiments did not seem to be particularly virus type dependent. However, it is not clear how these data are related to the ratios in natural surface waters, and we did observe considerable variation over time. Other assays, such as cell culture PCR, have been suggested for detection of viruses in source and tap waters (9), and the major drawback of such assays is that they do not provide quantitative data like plaque assays do. In addition, compared with a total culturable virus assay the multiplex integrated cell culture PCR was found to yield data that may over- or underestimate the amount of virus (16). Alternatively, other animal caliciviruses that can be grown on cells could serve as a model for human noroviruses. Another possibility is that outbreak data (12) or human volunteer studies (17) may be used to determine the ratio of infectious particles to defective particles, but to our knowledge such studies have not been conducted yet.

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